



# Fas/CD95/Apo-I activates the acidic sphingomyelinase via Caspases

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## Abstract

**Fas/CD95/Apo-I has been shown to stimulate a variety of molecules including several members of the caspase family and the acidic sphingomyelinase (Martin and Green 1995; Gulbins *et al*, 1995). Here, we demonstrate that Fas receptor-triggered activation of the acidic sphingomyelinase, consumption of sphingomyelin, release of ceramide, and subsequent activation of JNK and p38-K are regulated by caspases. Inhibition of caspases by Ac-YVAD-chloromethylketone or transient CrmA transfection prevented stimulation of acidic sphingomyelinase, release of ceramide and activation of JNK and p38-K upon Fas-receptor crosslinking. Likewise, Fas triggered apoptosis was almost completely blocked by Ac-YVAD-chloromethylketone or CrmA mediated inhibition of caspases. The results suggest a new signalling cascade from the Fas receptor via caspases to acidic sphingomyelinase, ceramide and JNK/p38-K.**

**Keywords:** apoptosis; T-lymphocytes; signalling; ceramide; proteolysis

**Abbreviations:** JNK, Jun N-terminal kinase; TNF, tumor necrosis factor; NGF, nerve growth factor; ASM, acidic sphingomyelinase; GST, glutathione S-transferase; I<sub>2</sub>-Iodine; PAGE, polyacrylamidgel

## Introduction

Apoptosis has been identified as an evolutionary conserved and fundamental active cellular mechanism occurring under a range of physiological and pathological conditions (Wyllie *et al*, 1980; Raff, 1992; Cohen, 1993). Programmed cell death is characterized by distinct morphological changes of the cell including nuclear condensation and fragmentation, membrane blebbing and formation of apoptotic bodies (Kerr *et al*,

1972). Several stimuli including the TNF-, NGF-, Fas/ApoI/CD95-receptor, reactive oxygen intermediates (ROI), ionizing radiation, ceramides, heat shock, cytotoxic drugs, infection by some viruses or bacteria or withdrawal of growth factors induce apoptosis in different cell types (Beutler and Huffer, 1994; Bose *et al*, 1995; Collins, 1995; Hockenberry *et al*, 1993; Itoh *et al*, 1991; Obeid *et al*, 1993; Oehm *et al*, 1992; Smith *et al*, 1994; Stevenson *et al*, 1994; Verheij *et al*, 1996; Xia *et al*, 1995).

The cellular homeostasis of the immune system is regulated by the balance between cell proliferation and programmed cell death. Apoptosis in mature lymphocytes can be induced by cellular stimulation via the Fas/ApoI/CD95-receptor (Singer and Abbas, 1994) belonging to the family of the NGF/TNF-receptors, which are important in the regulation of apoptosis, proliferation or differentiation (Smith *et al*, 1994; Beutler and Huffer, 1994). The major function of the Fas-receptor/Fas-ligand system seems to be the regulation of the peripheral immune response (Singer and Abbas, 1994; Nagata and Suda, 1995). Thus, mutations of the Fas receptor or its ligand result in the defects of *lpr* and *gld* mice, respectively, characterized by lymphadenopathy, lymphoaccumulation and autoimmune organ failure (Watanabe-Fukunaga *et al*, 1992; Takahashi *et al*, 1994; Wu *et al*, 1994).

Several components of the death machinery have been identified. In particular, proteases of the caspase-family have been shown to be important in Fas-triggered apoptosis (Los *et al*, 1995; Enari *et al*, 1995). Recently, it was demonstrated that the Fas receptor associates via its death domain, which exhibits significant homology with an intracellular domain of the TNF receptor and is required for induction of apoptosis (Tartaglia *et al*, 1993), with FADD, TRADD or RIP (Chinnaiyan *et al*, 1995; Hsu *et al*, 1995; Stanger *et al*, 1995). FADD in turn binds to caspase 8 (FLICE/MACH-1) (Boldin *et al*, 1996; Muzio *et al*, 1996). Caspase 8 contains an ICE/Ced-3-like protease domain which seems to be activated upon ligation and trimerization of Fas by a conformational change of caspase 8 (Boldin *et al*, 1996; Muzio *et al*, 1996). Caspase 8 then transmits the activation signal to other caspases, finally triggering cell death (Boldin *et al*, 1996; Muzio *et al*, 1996). We and others have previously demonstrated that acidic sphingomyelinases and ceramide also play an important role for Fas induced apoptosis (Cifone *et al*, 1994; Tepper *et al*, 1995; Gulbins *et al*, 1995). Ceramides are known stimuli of apoptosis and are released by activation of an acidic and/or neutral sphingomyelinase (Hannun, 1996). Both enzymes have been shown to be activated by the Fas receptor (Cifone *et al*, 1995; Tepper *et al*, 1995; Gulbins *et al*, 1995). The regulatory mechanisms of sphingomyelinase activation by the Fas receptor are completely unknown; however, recent data suggest that reaper triggered release of ceramide depends on the function of caspases (Pronk *et al*,

*al*, 1996), implying that sphingomyelinases and ceramide are downstream targets of caspases. Ceramide has been shown to stimulate a variety of enzymes including a phosphatase (Joseph *et al*, 1993), a ceramide activated proline-directed protein kinase (Dobrowsky *et al*, 1993), Jun-N-terminal kinase (Verheij *et al*, 1996; Westwick *et al*, 1995), Raf-K (Huwiler *et al*, 1996) and tyrosine phosphorylation (Ji *et al*, 1995).

Several other molecules have been shown to be involved in Fas-triggered apoptosis, in particular the small G-protein p21 Ras and inhibition of Ras blocks Fas-induced programmed cell death in Jurkat T cells (Gulbins *et al*, 1995). Further molecules regulated upon Fas receptor triggering include phospholipase A<sub>2</sub> (Cifone *et al*, 1995), NFκB (Ponton *et al*, 1996), Jun-N-terminal Kinases (Latinis and Koretzky, 1996), tyrosine and serine/threonine-kinases (Eischen *et al*, 1994; Schlottmann *et al*, 1996; Tian *et al*, 1995), the tyrosine phosphatase FAP (Sato *et al*, 1995), Rac-proteins (Gulbins *et al*, 1996) or the K<sub>v</sub>1.3 potassium-channel (Szabo *et al*, 1996).

To gain some insight into the molecular mechanisms of regulation of acidic sphingomyelinase, we investigated the effect of a pharmacological or CrmA mediated inhibition of caspases on acidic sphingomyelinase stimulation by Fas. The data provide evidence for a regulation of the acidic sphingomyelinase, consumption of sphingomyelin, release of ceramide and finally JNK/p38-K-activity by caspases, pointing to a novel signalling cascade important for Fas-induced apoptosis.

## Results

Several results suggest a crucial function of caspases and the acidic sphingomyelinase releasing ceramide in apoptosis (Cifone *et al*, 1994; Gulbins *et al*, 1995; Tepper *et al*, 1995; Pronk *et al*, 1996). The transient and early activation of some caspases by the Fas receptor as well as the involvement of caspases in reaper-mediated ceramide release points to a possible signalling function of caspases (Los *et al*, 1995; Enari *et al*, 1995; Pronk *et al*, 1996) in addition to being executors of cell death.

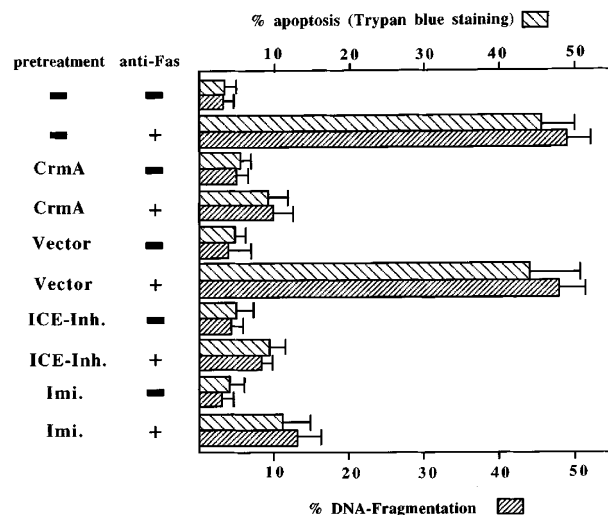
To test the hypothesis that caspases are involved in the regulation of ceramide release via stimulation of acidic sphingomyelinase, we used two inhibitors of caspases. The pharmacological peptide Ac-YVAD-chloromethylketone inhibits predominantly caspase-1 (Darmon *et al*, 1996). Second, we transiently transfected a serpin like inhibitor of caspases, the viral gene CrmA, together with the B-cell surface antigen CD20 and sorted transfected cells via CD20. Similar to the pharmacological inhibitor, CrmA predominantly inactivates caspase-1, but at higher concentrations also caspase-3 (Nicholson *et al*, 1995; Tewari *et al*, 1995).

Treatment of Jurkat T cells with Ac-YVAD-chloromethylketone or CrmA prevented Fas induced cell death, showing the efficiency of caspase inhibition (Figure 1). Next, we determined the consumption of sphingomyelin upon Fas receptor ligation in cells treated with Ac-YVAD-chloromethylketone, transfected with CrmA, or left untreated. The results show that pharmacological

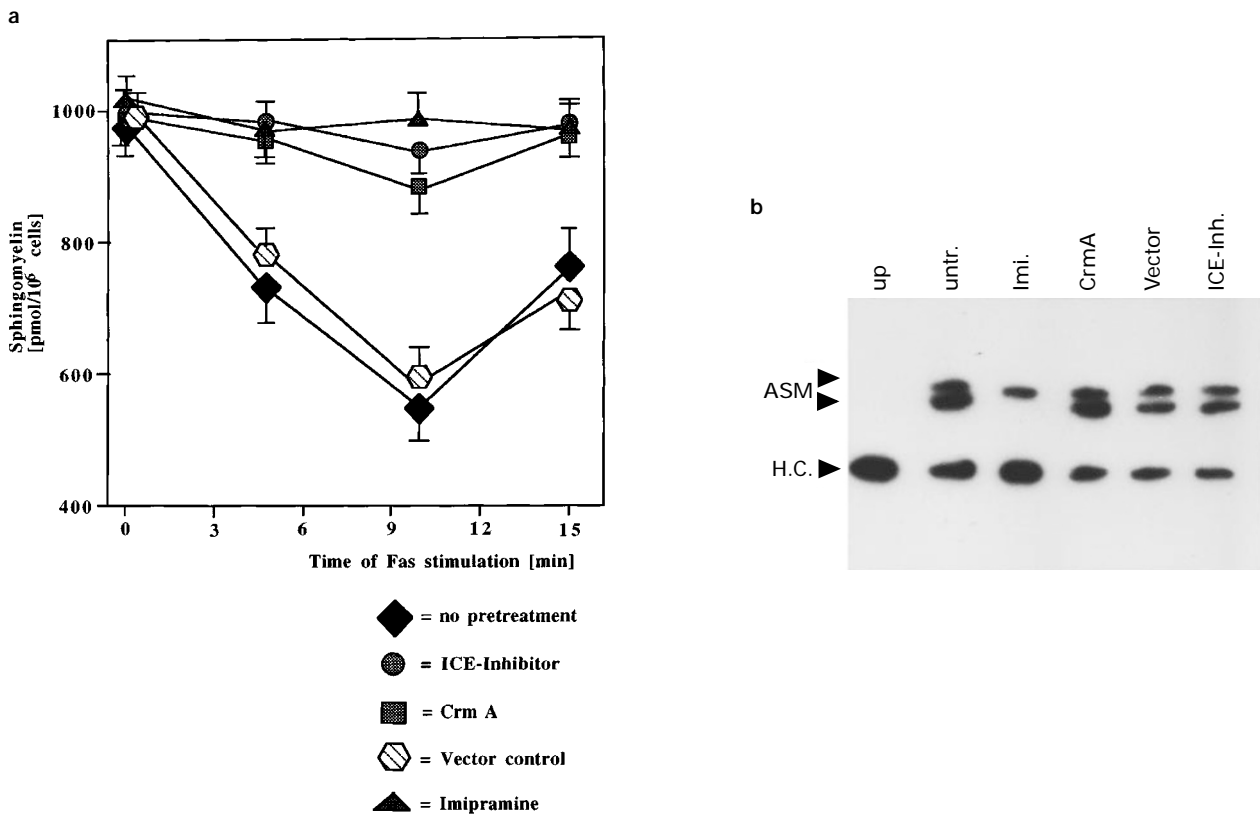
or CrmA mediated inhibition of caspases almost completely prevents sphingomyelin consumption after Fas receptor triggering in untreated cells (Figure 2a). In addition, we directly inhibited acidic sphingomyelinase using the drug imipramine (40 μM). Imipramine induces degradation of the active 72 kDa isoform of the ASM by an unknown mechanism, whereas the inactive 75 kDa proform of the ASM is not affected by this drug (Hurwitz *et al*, 1994). Efficient downregulation of the ASM upon incubation with imipramine was demonstrated by Western blotting of ASM immunoprecipitates from imipramine-treated or untreated Jurkat T cells (Figure 2b). Shorter incubation with (less than 30 min) or lower doses (10 or 20 μM) of imipramine resulted in a partial reduction of acidic sphingomyelinase expression correlating with a partial inhibition of acidic sphingomyelinase activity and cell death (not shown).

Imipramine treated resulted in an almost complete abrogation of sphingomyelin consumption upon Fas ligation (Figure 2a). In accordance with the inhibition of sphingomyelin consumption, transfection with CrmA or treatment of the Jurkat cells with Ac-YVAD-chloromethylketone prevented the release of ceramide detected in normal, untreated Jurkat cells upon Fas receptor stimulation (Figure 3a and b). Ceramide release was also greatly reduced by preincubation of the cells with imipramine which directly blocks acidic sphingomyelinase (Figure 3a and b).

Inhibition of sphingomyelin consumption and ceramide release by pharmacological or CrmA mediated blockade of caspases correlated with an almost complete prevention of Fas induced acidic sphingomyelinase activation (Figure



**Figure 1** Fas induced cell death is inhibited by transient transfection of CrmA or pharmacological inhibition of caspases. Jurkat cells were transiently transfected with CrmA or a control vector, labeled with [<sup>3</sup>H]thymidine and stimulated via Fas. Alternatively, labeled cells were treated with 10 μM Ac-YVAD-chloromethylketone for 60 min prior to stimulation with anti-Fas. After 3 h incubation, cells were collected and DNA fragmentation determined by filtration through glass fiber filters and liquid scintillation counting. An aliquot of the cells was trypan blue stained and analyzed for typical morphological changes of apoptosis (Imi.=imipramine, ICE-Inh.=ICE-inhibitor, vector=vector control). Experiments were done in triplicate and repeated three times



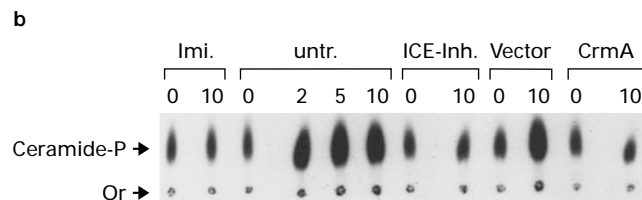
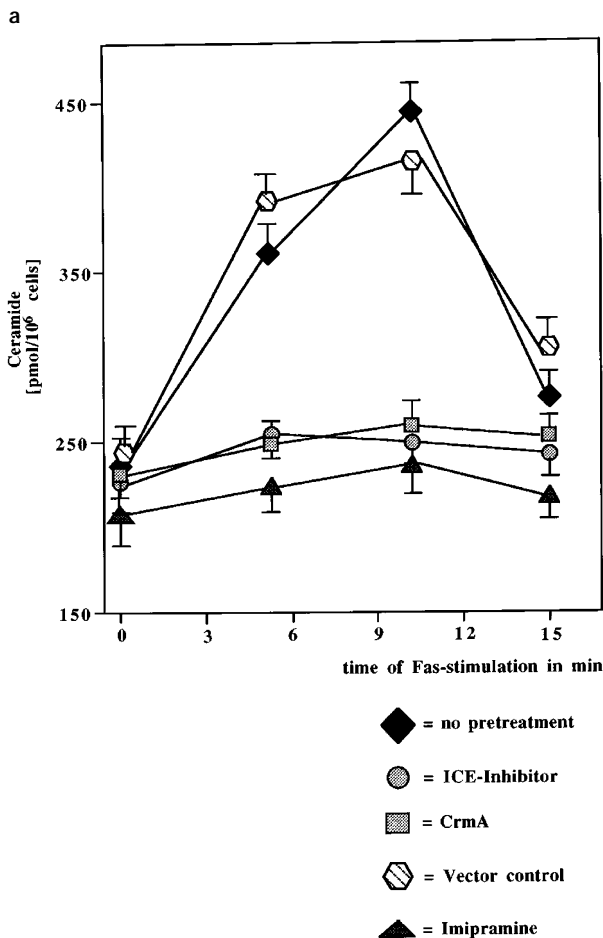
**Figure 2** (a) Genetic or pharmacological inhibition of caspases prevents Fas induced sphingomyelin consumption. Similar, imipramine-induced acidic sphingomyelinase degradation blocks. Fas-triggered sphingomyelin hydrolysis. Jurkat T lymphocytes were transiently transfected with CrmA or vector control and an expression vector for CD20. Transfected or untransfected cells were labeled for 12 h with [<sup>3</sup>H]choline chloride, pretreated for 60 min with Ac-YVAD-chloromethylketone (10  $\mu$ M) or imipramine (40  $\mu$ M) or left untreated and stimulated via Fas. Cells were extracted organically and the extracts separated by TLC. Sphingomyelin spots were identified by comigration with sphingomyelin standards upon incubation of the TLC plates in I<sub>2</sub>-vapor. Spots were scraped from the plates and analyzed by liquid scintillation counting. Data are expressed as mean  $\pm$  s.d. of three independent experiments. (b) shows the reduction of acidic sphingomyelinase expression induced by imipramine. Acidic sphingomyelinase was immunoprecipitated from untreated (untr.) imipramine- or Ac-YVAD-chloromethylketone-treated and CrmA- or control-transfected cells. Immunoprecipitates were separated by 10% SDS-PAGE, blotted, incubated with a polyclonal anti-acidic sphingomyelinase antiserum and developed with horseradish-coupled protein G and ECL. Unspecific immunoprecipitates (up) were performed with an irrelevant goat antiserum. (H.C.=heavy chain of the anti-acidic sphingomyelinase-antibody used for immunoprecipitation)

4a), whereas untreated cells responded to anti-Fas with an approximately threefold stimulation of acidic sphingomyelinase activity. Acidic sphingomyelinase activity was directly measured by immunoprecipitation of acidic sphingomyelinase from stimulated or unstimulated cells and subsequent enzyme assay. Cells were either pretreated with the peptide Ac-YVAD-chloromethylketone blocking caspases or transfected with CrmA 36 h prior to the assay. Imipramine reduced acidic sphingomyelinase activity (Figure 4) and protein levels by approximately 95% (Figure 2b), however, the remaining 5% of acidic sphingomyelinase responded normally with a threefold stimulation of acidic sphingomyelinase activity. To further confirm that treatment of the cells with imipramine does not render the cells unresponsive to Fas-receptor ligation we determined the release of DAG upon Fas receptor ligation, which has been shown to be activated independently of the ASM after TNF receptor ligation (Schütze *et al*, 1992). The results (Figure 4b) demonstrating a significant increase of

DAG-release after Fas show that treatment of Jurkat cells with imipramine does not induce a general signalling block. This is also indicated by the finding that addition of 10  $\mu$ M C6-ceramide to imipramine treated cells induced a similar rate of apoptosis as observed in cells not treated with imipramine (51  $\pm$  4% after 4 h in imipramine treated vs 53  $\pm$  6% in untreated cells).

A similar inhibition of Fas induced cell death and activation of the acidic sphingomyelinase was detected after incubation of Jurkat cells with 50  $\mu$ M ZVAD-fmk (not shown) further supporting the notion of acidic sphingomyelinase regulation by caspases. The release of ceramide has been previously linked to the activation of JNK upon stimulation of cells via the Fas- or TNF-receptor or treatment with X-ray, UV-light or heat shock (Verheij *et al*, 1996). We, therefore, tested, whether caspases are involved in the activation of JNK or p38-K by Fas (Figure 5). JNK or p38-K activity was determined by incorporation of <sup>32</sup>P<sub>i</sub> in the substrate GST-c-Jun or GST-ATF-2,

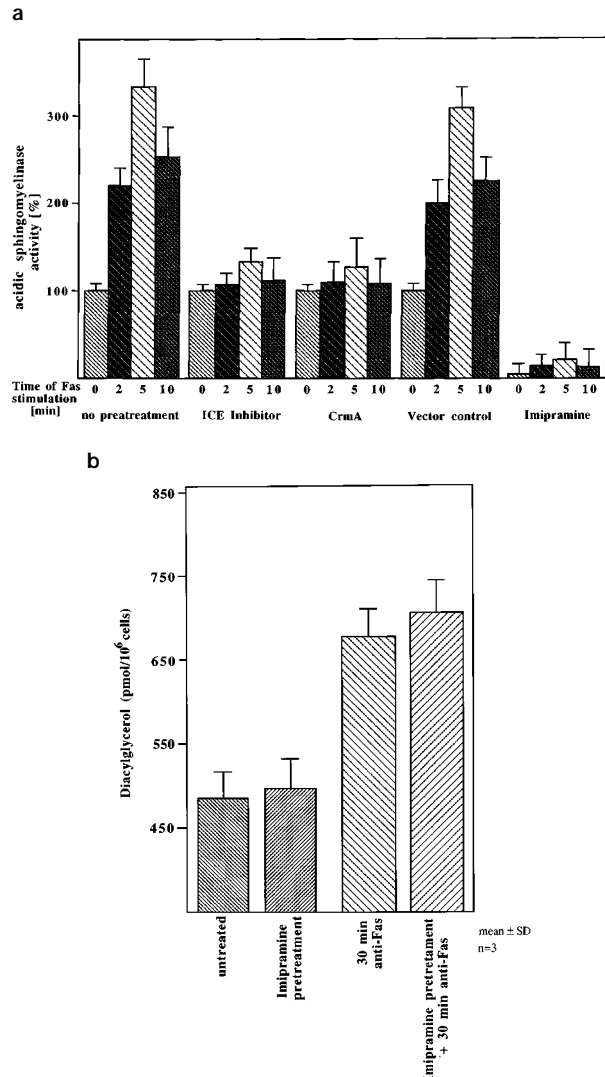
respectively. Figure 5 shows that the approximately tenfold activation of JNK or p38-K triggered by Fas is almost completely prevented by inhibition of caspases after pretreatment with Ac-YVAD-chloromethylketone or transfection of CrmA. Further, pretreatment of Jurkat cells with imipramine abolished the activation of JNK and p38-K by the Fas-receptor. The data point to a signalling cascade from Fas via caspases to ASM, ceramide and finally JNK/p38-K activation (Figure 6).



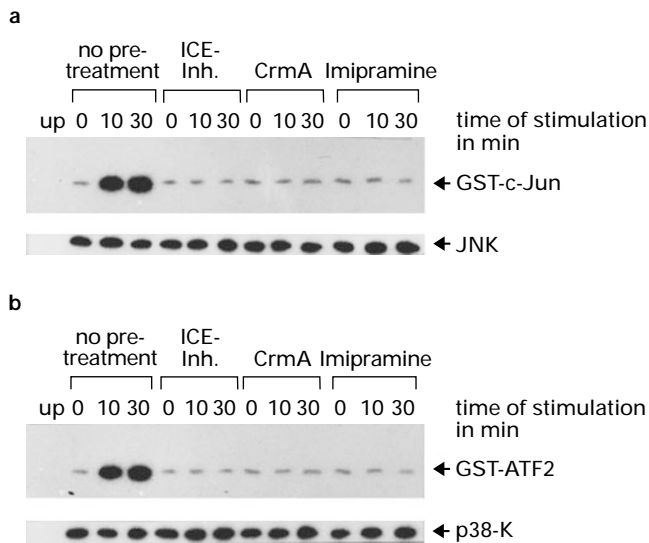
**Figure 3** Ceramide release upon Fas receptor ligation is inhibited by genetic or pharmacological inhibition of caspases or acidic sphingomyelinase. CrmA or control transfected, Ac-YVAD-chloromethylketone- or imipramine-treated cells were stimulated with anti-Fas (2 µg/ml) for the indicated time or left untreated. Cells were extracted, incubated with a ceramide-sensitive kinase in the presence of [<sup>32</sup>P];ATP. Samples were again extracted organically, dried, separated by TLC (silica G60) and analyzed by autoradiography and liquid scintillation counting. Shown is the mean ± s.d. of three independent results (a) and a representative autoradiography (b)

## Discussion

In the present study we provide evidence for a new signalling pathway from Fas via caspases to the activation of acidic



**Figure 4** (a) Activation of acidic sphingomyelinase by Fas is regulated by caspases. Genetic or pharmacological inhibition of caspases prevents Fas mediated acidic sphingomyelinase stimulation. The direct acidic sphingomyelinase inhibitor imipramine reduces the absolute values of acidic sphingomyelinase activity by approximately 95%. Acidic sphingomyelinase was immunoprecipitated from Fas-stimulated or unstimulated Jurkat cells transiently transfected with CrmA or vector control or treated with Ac-YVAD-chloro-methylketone or imipramine, respectively. Acidic sphingomyelinase immunoprecipitates were incubated with [<sup>14</sup>C]sphingomyelin for 30 min and the degradation of [<sup>14</sup>C]sphingomyelin was determined by liquid scintillation counting after organic extraction of the samples. Shown are changes of acidic sphingomyelinase activity compared to the value from unstimulated and untreated cells. Given are the mean ± s.d. from three independent experiments. (b) Imipramine treatment does not inhibit the release of DAG upon Fas receptor ligation. Imipramine treated or untreated cells were stimulated for 30 min via Fas as described above, DAG was extracted from the lysates and samples were incubated with a DAG sensitive bacterial kinase in the presence of [<sup>32</sup>P];ATP. Samples were dried, resuspended, separated by TLC and analyzed by autoradiography followed by liquid scintillation counting of the corresponding spots

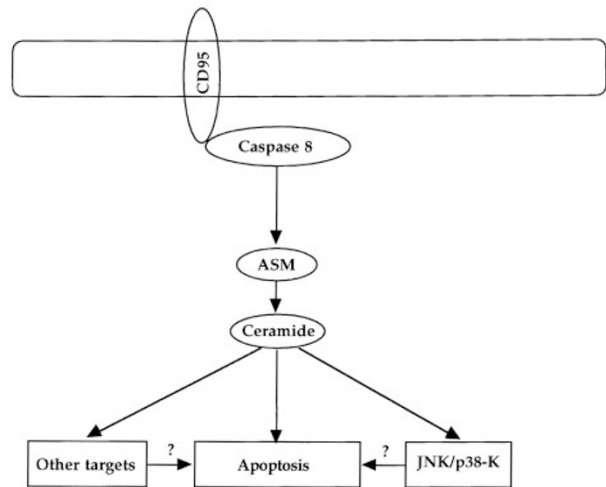


**Figure 5** Inhibition of caspases or the acidic sphingomyelinase by genetic or pharmacological techniques prevents the activation of JNK (a) or p38-K (b) by Fas-receptor ligation. CrmA transfected, Ac-YVAD-chloromethylketone or imipramine treated cells were stimulated via Fas, lysed and JNK or p38-K were immunoprecipitated from the lysates using polyclonal rabbit anti-JNK or anti-p38-K antibodies. Immunoprecipitates were incubated for 15 min with [<sup>32</sup>P];ATP (10 μCi/sample) and GST-c-Jun or GST-ATF-2, samples were separated by SDS-PAGE, blotted and analyzed by autoradiography. Unspecific immunoprecipitates (up) were performed from anti-Fas stimulated (10 min) cells using an irrelevant, affinity purified rabbit antibody. An aliquot of the immunoprecipitates was blotted with anti-JNK or anti-p38-K to analyze protein levels in all lanes (small blots). Shown are representative autoradiographies of three independent experiments each

sphingomyelinase and the formation of ceramide. Using genetic and pharmacological inhibition of caspases we show that caspases are upstream regulators of the acidic sphingomyelinase linking the Fas receptor to the release of ceramide.

Acidic sphingomyelinase is synthesized in the cells as an inactive 75 kDa isoform which is then processed to the active 72 kDa isoform (Hurwitz *et al*, 1994; Lansmann *et al*, 1996). The molecular details of this processing step are unknown. It is tempting to speculate that caspase-1 or other caspases cleave acidic sphingomyelinase and thus activate the enzyme. However, this hypothesis seems to be unlikely since known caspases are cytosolic whereas acidic sphingomyelinase resides in the endosomal/lysosomal compartment. Therefore, an indirect mechanism involving further enzymes between caspases and acidic sphingomyelinase mediating the activation of acidic sphingomyelinase is more likely. Since proteins are usually unable to cross the lipid bilayer separating caspases and acidic sphingomyelinase, it seems possible that further lipid metabolites, which readily flip from one to the other compartment, are released in the cytosol upon Fas receptor ligation and mediate the activation of the acidic sphingomyelinase.

CrmA and Ac-YVAD-chloromethylketone have been previously shown to inhibit members of the caspase family and to prevent Fas-induced apoptosis (Smith *et al*, 1996; Tewari and Dixit, 1995) pointing to the significance of this



**Figure 6** Model of Fas regulated activation of acidic sphingomyelinase by caspases. Fas may stimulate several members of the caspases family including caspase 8, which then directly or indirectly stimulate the acidic sphingomyelinase. Ceramide released by acidic sphingomyelinase activity may stimulate other members of the caspase family, JNK/p38-K or other molecules involved in Fas receptor signalling

signalling pathway for Fas mediated apoptosis. CrmA predominantly inhibits caspase-1 and -8, however, at very high concentrations it also binds to and inhibits caspase-3 (Nicholson *et al*, 1995; Tewari *et al*, 1995; Salvesen *et al*, 1997). Similar to CrmA, the pharmacological inhibitor Ac-YVAD-chloromethylketone inhibits predominantly caspase-1, whereas several other caspases are almost unaffected by this peptide (Darmon *et al*, 1996). Since caspase-8 seems to be one of the primary cellular targets of CrmA under physiological conditions (Zhou *et al*, 1997) and plays a major role in Fas triggered cell death (Boldin *et al*, 1996; Muzio *et al*, 1996), this caspase is a good candidate for the protease involved in the regulation of acidic sphingomyelinase activation by Fas. However, the exact nature of the caspase involved in acidic sphingomyelinase activation has to be elucidated.

Our data suggest that caspase-1 itself or enzymes closely related to caspase-1 are (indirectly) mediating the activation of acidic sphingomyelinase upon Fas receptor ligation. It is unknown whether caspases are only executors of apoptosis or whether they also function as signalling molecules, however, our data indicate a signalling function of at least some caspases rather than being just executors of apoptosis. This notion is supported by several other studies: First, expression of the reaper protein, which is homologous to the cytoplasmic domain of Fas or TNF results in activation of caspases as well as in the formation of ceramide (Pronk *et al*, 1996). Inhibition of caspases prevents reaper triggered ceramide release placing ceramide downstream of caspases (Pronk *et al*, 1996). Ceramide, however, stimulates via a Bcl2 sensitive pathway other members of the caspase family, in particular caspase-3 (Smyth *et al*, 1996; Zhang *et al*, 1996). Thus,



ceramide seems to be an intermediate in the death cascade between several caspases. Second, expression of CrmA in transgenic mice prevents Fas-induced apoptosis (Smith *et al*, 1996). However, these lymphocytes are still susceptible to X-irradiation induced apoptosis. Since X-rays induce an increase of ceramide concentration (Haimovitz-Friedman *et al*, 1994), the results of these transgenic animals suggest that ceramide functions downstream of at least some caspases to mediate cell death. Third, it has been recently shown, that CrmA-, YVAD-cmk- or Z-VAD-fmk-mediated inhibition of caspases prevents Fas triggered p38-K activation (Juo *et al*, 1997) directly supporting the data of the present study. Further, Fas-receptor ligation has been shown to induce only a transient activation of caspases, peaking approximately already 20 min after stimulation via the Fas receptor (Los *et al*, 1995; Enari *et al*, 1995). Thus, some caspases seem to be very early signalling molecules activated by the Fas receptor and might be upstream of the acidic sphingomyelinase. Finally, the release of ceramide upon ligation of the TNF receptor belonging to the same receptor family as Fas is inhibited by transfection of CrmA (Dbaibo *et al*, 1997). This also suggests that some CrmA sensitive caspases are involved in the regulation of a sphingomyelinase.

Our notion of an activation of JNK and p38-K by Fas receptor ligation via stimulation of the acidic sphingomyelinase is supported by previous studies showing that Fas or ceramides stimulate JNK/p38-K (Verheij *et al*, 1996; Westwick *et al*, 1995; Latinis *et al*, 1996; Juo *et al*, 1997; Lenczowski *et al*, 1997). Furthermore, ceramide-initiated JNK activation has been demonstrated to be essential for the induction of apoptosis after ionizing radiation, UV-light, heat shock, TNF $\alpha$  or H<sub>2</sub>O<sub>2</sub> (Verheij *et al*, 1996), suggesting an important function of ceramide triggered activation of JNK for ceramide mediated programmed cell death. Fas belongs to a family of receptors including TNF, p75-NGF, OX-40, CD40 and some further molecules (Beutler and Huffel, 1994; Smith *et al*, 1994). Apoptosis induced by p75-NGF receptor has been shown to involve activation of JNK/p38-K and inhibition of JNK/p38-K prevented cell death induced by NGF withdrawal from PC12-pheochromocytoma cells (Xia *et al*, 1995). The activation of a similar signal transduction pathway has been reported to be stimulated by the TNF receptor (Verheij *et al*, 1996; Schütze *et al*, 1992; Trent *et al*, 1996). These studies show a release of ceramide and an activation of Ras and JNK/p38-K after TNF receptor ligation which are critically involved in TNF-receptor mediated cell death (Verheij *et al*, 1996; Schütze *et al*, 1992; Trent *et al*, 1996). Thus, TNF, p75-NGF and Fas seem to activate similar molecular mechanisms upon induction of apoptosis. However, a recent study using transfection of FADD, RIP or TRAF-2 into MCF-7 cells proposed that JNK-activation and apoptosis after TNF-receptor ligation are separate responses (Liu *et al*, 1996). Therefore, JNK-activation may not be sufficient to trigger apoptosis by itself, however, under physiological conditions it might be necessary as a positive signal for other signals triggering programmed cell death. In situations with very high activation levels of the pathway triggering apoptosis, e.g. after transfection of

genes inducing cell death, this positive feedback may not be absolutely required.

The results of the present study using imipramine point to an involvement of the acidic sphingomyelinase in Fas triggered death. However, since imipramine probably does not specifically inhibit the acidic sphingomyelinase, the exact function of this enzyme in Fas triggered cell death has to be elucidated using genetic approaches.

Taken together, the data suggest the following model: Fas activates some caspases (caspase 8 is a good candidate) which then stimulate directly or indirectly the acidic sphingomyelinase resulting in the release of ceramide. The activation of acidic sphingomyelinase and the release of ceramide seem to be crucial for Fas induced apoptosis since acidic sphingomyelinase deficient lymphocytes are resistant to Fas induced apoptosis (R. Kolesnick, personal communication). Ceramide may have multiple functions including the activation of JNK/p38-K as well as the stimulation of further members of the caspase family, e.g. caspase-3. These molecules then execute the apoptotic signal delivered by cellular stimulation via the Fas receptor (Figure 6).

## Materials and Methods

### Cell culture and stimulation

All reagents were purchased from Sigma, Deisenhofen, Germany, if not otherwise cited. Human Jurkat T cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate, 100  $\mu$ M non essential amino acids, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 50  $\mu$ M  $\beta$ -mercaptoethanol (all from Gibco-BRL, Eggenstein, Germany). For activation, cells were washed twice in HEPES buffered saline (H/S, 132 mM NaCl, 20 mM HEPES, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 0.7 mM MgCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>) and incubated at 37°C with 2  $\mu$ g/ml monoclonal anti-human-Fas-antibody (clone CH-11; Dianova-Immunotech, Hamburg, Germany) for the indicated times.

### Inhibition of caspases and acidic sphingomyelinase

Co-transfection of Jurkat cells with 40  $\mu$ g each SRS $\alpha$ -*crmA* or vector control (SRS $\alpha$ ) with an expression vector for CD20 (pRc/CMV-*cd20*) (10  $\mu$ g) was performed as previously described (10). Briefly, cells were electroporated using a BTX-electroporator at five pulses (99  $\mu$ sec) and 500 V. Twelve hours later viable cells were purified by Ficoll gradient centrifugation and cultured for an additional 24 h. CD20<sup>+</sup> cells were then selected by incubation with 50  $\mu$ g/ml anti-CD20 mAb (Dianova) (60 min, 4°C), three times washing and further incubation (60 min, 4°C) with magnetic beads coated with a sheep anti-mouse Immunoglobulin (Dynal, Hamburg, Germany) (Gulbins *et al*, 1995). Since the ratio of 5:1 SRS $\alpha$ -*crmA*:pRc/CMV-*cd20* drives expression of CrmA in any CD20<sup>+</sup> cell, the selection of CD20<sup>+</sup> cells permits effective sorting for CrmA expressing cells. The fraction of CD20-positive and thus CrmA transfected cells was determined by *in vivo* labeling of the cells with [<sup>3</sup>H]thymidine as previously described (Gulbins *et al*, 1995). Aliquots of labeled cells were counted prior and after sorting via magnetic beads. These experiments showed that approximately 10% of all cells were CD20-positive. Non-specific binding of the cells to the magnetic beads did not exceed 0.5% of all

cells as determined by incubation of cells with an irrelevant monoclonal mouse antibody (50  $\mu\text{g}$ ) and magnetic beads. Purified cells were allowed to recover for 30 min at 37°C and then used for biological assays or determination of apoptosis.

Alternatively, caspases were inhibited by 60 min preincubation with 10  $\mu\text{M}$  of the irreversible pharmacological inhibitor Ac-YVAD-chloromethylketone (Bachem, Hannover, Germany). Acidic sphingomyelinase was inhibited by 60 min preincubation of the cells with 40  $\mu\text{M}$  imipramine in RPMI/1640 supplemented with 0.5% BSA, 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate and 100  $\mu\text{M}$  non essential amino acids.

### Measurement of sphingomyelin consumption

Cells were metabolically labeled by incubation with 1  $\mu\text{Ci/ml}$  [methyl- $^3\text{H}$ ]choline chloride (60–90 Ci/mmol; NEN-DuPont, Bad-Homburg, Germany) for 48 h in complete RPMI-medium. Cells were washed three times and stimulated via Fas for the indicated time. Infection was terminated by extraction in 120  $\mu\text{l}$  0.22 M HCl, 2.7 ml  $\text{CHCl}_3:\text{CH}_3\text{OH}$  (2:1, v/v), 0.9 ml of  $\text{CHCl}_3$  and 0.9 ml of 1 M KCl. The organic phase was collected after mixing and centrifugation for 15 min at 800  $\times$  g, dried, resuspended in  $\text{CHCl}_3:\text{CH}_3\text{OH}$  (1:1, v/v) and lipids were separated on G60 silica gel thin layer chromatography plates (Machery-Nagel, Dueren, Germany) using  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}:\text{acetic acid}$  (50:30:8:4, v/v/v/v) as solvent system. Sphingomyelin was identified by co-migration with standards, scrapped from the plate and radioactivity in each spot determined by liquid scintillation counting.

### Activity of acidic sphingomyelinase

The activity of acidic sphingomyelinase upon Fas receptor ligation was measured by an immunoprecipitation technique. Stimulated or unstimulated cells were lysed in ice-cold 50 mM Tris (pH 7.4), 10 mM Bacitracin, 1 mM Benzamidine, 10 mM  $\text{Na}_3\text{VO}_4$ , 10  $\mu\text{g/ml}$  of each aprotinin and leupeptin, 0.1 mg/ml soybean trypsin inhibitor and 0.2% Triton X-100 and immediately sonicated for 10 s. Sonication was repeated three times and insoluble cell debris pelleted by 5 min centrifugation at 600  $\times$  g. An equal amount of 50 mM Tris (pH 7.4), 3% NP40, 1% Triton X-100, 10 mM  $\text{Na}_3\text{VO}_4$ , 100  $\mu\text{g/ml}$  each aprotinin and leupeptin (lysis buffer) was added to the samples and ASM was immunoprecipitated from the supernatants using a goat anti-ASM-antiserum previously characterised (Hurwitz *et al*, 1994). Samples were incubated for 4 h at 4°C, immunocomplexes were immobilized by addition of 20  $\mu\text{l}$  protein A/G coupled agarose (Santa Cruz Inc, Santa Cruz, CA), and further incubation for 60 min at 4°C. The immunoprecipitates were washed three times in lysis buffer, followed by additional three washes in 50 mM sodium acetate (pH 5.0), 0.2% Triton X-100, 1 mM  $\text{Na}_3\text{VO}_4$  and 10  $\mu\text{g/ml}$  each aprotinin and leupeptin. Washed immunoprecipitates were incubated with [ $^{14}\text{C}$ ]sphingomyelin (0.5  $\mu\text{Ci/sample}$ , 54.5 mCi/mmol; NEN-DuPont) in 250 mM sodium acetate (pH 5.0), 1.3 mM EDTA, 0.05% NP40 (assay buffer) at 37°C for 30 min. The substrate [ $^{14}\text{C}$ ]sphingomyelin was solubilized after drying by 10 min bath sonication in assay buffer. Assays were terminated by extraction with 800  $\mu\text{l}$  Chloroform:Methanol (2:1, v/v) and 250  $\mu\text{l}$   $\text{H}_2\text{O}$ . The upper phase was collected and radioactivity reflecting the degradation of [ $^{14}\text{C}$ ]sphingomyelin was determined by liquid scintillation counting.

### Determination of diacylglycerol and ceramide

For determination of ceramide, cells were stimulated with anti-Fas antibodies for the indicated time, washed and extracted with  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{HCl}$  (100:100:1, v/v/v). Aliquots of the organic phase

were dried under vacuum, DAG was digested by mild alkaline hydrolysis in 0.1 M methanolic KCl, the samples were reextracted as above, dried and solubilized by 10 min bath sonication in 7.5% (w/v) *n*-octyl- $\beta$ -glucopyranoside, 5 mM cardiolipin, 1 mM diethylenetriamine-pentaacetic acid (DETAPAC). After sonication 40  $\mu\text{g/ml}$  of purified *E. coli* DAG-kinase (Amersham, Braunschweig, Germany) in 70  $\mu\text{l}$  reaction buffer (100 mM imidazole-HCl (pH 6.6), 100 mM NaCl, 25 mM  $\text{MgCl}_2$  and 2 mM EGTA) supplemented with 10  $\mu\text{Ci/sample}$  [ $^{32}\text{P}$ ] $\gamma$ -ATP and 1 mM ATP were added and the samples were incubated at 22°C for 30 min. The kinase reaction was stopped by addition of 1 ml  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{HCl}$  (100:100:1, v/v/v), 170  $\mu\text{l}$  of salt solution (10 mM HEPES (pH 7.2), 135 mM NaCl, 1.5 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$  and 5.6 mM glucose) and 30  $\mu\text{l}$  of 100 mM EDTA. The lower organic phase was dried, resuspended in  $\text{CHCl}_3:\text{CH}_3\text{OH}$  (1:1, v/v) and resolved by TLC using  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{acetic acid}$  (65:15:5, v/v/v) as solvent. Spots were visualized by autoradiography, scrapped from the plate and analyzed by liquid scintillation counting. The ceramide content of the samples was determined by comparison with a standard curve containing known quantities of ceramide.

The release of DAG was determined by the same protocol with omission of the mild alkaline hydrolysis in 0.1 M methanolic KCl.

### JNK- and p38-K-activity

To measure the activity of JNK or p38-K, cells were lysed in 25 mM HEPES (pH 7.4), 0.2% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl, 10 mM each NaF,  $\text{Na}_3\text{VO}_4$  and sodium pyrophosphate and 10  $\mu\text{g/ml}$  of each aprotinin and leupeptin (RIPA-buffer) after stimulation via Fas. Lysates were centrifuged at 25 000  $\times$  g for 20 min and JNK or p38-K immunoprecipitated from the supernatants at 4°C for 4 h using polyclonal rabbit anti-human JNK- or p38-K-antiserum (Santa Cruz Inc.). Immunocomplexes were immobilized on agarose-coupled protein A/G, incubated for additional 60 min at 4°C, washed twice in RIPA-buffer, twice in H/S, 1% NP-40, 2 mM  $\text{Na}_3\text{VO}_4$ , once in 100 mM TrisHCl (pH 7.5), 0.5 M LiCl and finally twice in kinase buffer 12.5 mM MOPS (pH 7.5), 12.5 mM  $\beta$ -glycerophosphate, 7.5 mM  $\text{MgCl}_2$  and 0.5 mM of each NaF, EGTA and  $\text{Na}_3\text{VO}_4$ . After washing, the immunoprecipitates were resuspended in kinase buffer supplemented with 10  $\mu\text{Ci/sample}$  [ $^{32}\text{P}$ ] $\gamma$ -ATP (6000 Ci/mmol, NEN/DuPont), 10  $\mu\text{M}$  ATP and 1  $\mu\text{g/ml}$  GST-c-Jun (aminoacids 1–79) or GST-ATF-2 (aminoacids 1–96). The samples were incubated at 30°C for 15 min and stopped by addition of 5  $\mu\text{l}$  boiling 5  $\times$  reducing SDS-sample buffer. Samples were separated by 10% SDS-PAGE and analyzed by autoradiography. The substrates GST-c-Jun or GST-ATF-2 were expressed in DH5 $\alpha$ -bacteria after transformation, growth for 12 h and incubation with IPTG (200  $\mu\text{M}$ ) for 4 h. Bacteria were lysed in 25 mM HEPES (pH 7.4), 0.2% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl and 10  $\mu\text{g/ml}$  of each aprotinin and leupeptin; GST-fusion proteins were purified by binding to glutathione agarose and eluted in kinase buffer with 20 mM glutathione. Purity of the preparations was tested by SDS-PAGE and Coomassie staining.

### Apoptosis

Fas-induced cell death was determined by metabolic labeling of CrmA-transfected or untransfected Jurkat cells for 12 h with 10  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]thymidine (8.3 Ci/mmol; NEN/DuPont) (Gulbins *et al*, 1995). Cells were washed, aliquoted, pretreated with Ac-YVAD-chloromethylketone or imipramine and incubated with anti-Fas (200 ng/ml) or left untreated. Cell death was determined after 3 h by DNA fragmentation and trypan blue staining. Briefly, cells were disrupted by one cycle of freezing at –20°C and thawing, unfragmented genomic DNA was

collected by filtration through glass fiber filters (Pharmacia, Freiburg, Germany) and counted by liquid scintillation (Gulbins *et al*, 1995). Results are expressed as % DNA fragmentation  $\pm$  s.d. compared with control samples. Experiments were done in triplicates and repeated three times. In all experiments an aliquot of the cells was analyzed for cell death by trypan blue. Apoptosis was further identified by typical morphological changes, in particular membrane blebbing, condensation and fragmentation of nuclei. Comparison of the DNA fragmentation method with morphologic changes permits exact determination of apoptosis vs necrosis. In all experiments apoptosis in untreated cells did not exceed 5% of all cells.

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